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Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by indirect ELISA

MARTINEZ (D.), COISNE (S.), SHEIKBOUDOU (C.), JONGEJAN (F.). Détection d'anticorps contre *Cowdria ruminantium* dans le sérum de ruminants domestiques par ELISA indirect. *Revue Élev. Méd. vét. Pays trop.*, 1993, 46 (1-2) : 115-120

Un test ELISA (enzyme-linked immunosorbent assay) a été mis au point pour la détection d'anticorps contre *Cowdria ruminantium* dans le sérum de ruminants domestiques. Les micro-organismes cultivés dans des cellules bovines endothéliales ombilicales ont été utilisés comme antigène. Lorsque la culture a montré une lyse à 90 p.100, le surnageant a été centrifugé, soniqué et appliqué sur des plaques microtitres en polystyrène. Des anticorps ont été détectés à partir de 9 jours après immunisation expérimentale de chèvres. La sensibilité de l'ELISA, calculée sur 73 sérums de ruminants, se trouvait entre 97,3 et 98,6 p.100. La spécificité globale du test était de 97 p.100 (N = 2925). Néanmoins, la spécificité était beaucoup plus basse pour les ovins (94,4 p.100, N = 881) que pour les caprins (98,6 p.100, N = 651) et les bovins (97,8 p.100, N = 1393). Des réactions croisées, qui peuvent expliquer certaines des réactions faussement positives, ont été trouvées entre l'antigène de *Cowdria* et des sérums contre *Ehrlichia bovis* (1 bovin positif sur 2 infectés expérimentalement) et *E. ovina* (2 moutons positifs sur 2 infectés), mais non pas contre *E. phagocytophila*. Les variabilités intra- et inter-test étaient respectivement de 7,5 et de 7,8 p.100, ce qui montre une bonne reproductibilité de l'ELISA.

Mots clés : Ruminant - Bovin - Caprin - Ovin - *Cowdria ruminantium* - Anticorps - Test ELISA - Antigène - Sérum - *Ehrlichia bovis* - *Ehrlichia ovina* - *Ehrlichia phagocytophila*.

INTRODUCTION

The development of serodiagnostic tests for cowdriosis has long been hampered by the inability to cultivate *Cowdria ruminantium* *in vitro*. A capillary flocculation test (13) and a complement fixation test (5) using crude extracts of brains from infected animals lacked sensitivity. An indirect fluorescent antibody test (IFAT) was described by DU PLESSIS (6) who used mouse peritoneal macrophages infected with a mouse infective stock of *Cowdria* (Kümm stock) as antigen. The use of primary blood neutrophil cultures as antigen by Logan (21) improved the specificity of the IFAT. The detection of antibodies to *Cowdria* in ruminant sera by ELISA using parasites purified from infected ticks, tissues or blood by chromatography techniques (33) or density gradient centrifugation (28)

was also reported. However, the unavailability of sufficient amounts of purified antigens by these techniques resulted in a poor sensitivity of the ELISA.

The cultivation of *Cowdria* in bovine endothelial cells *in vitro* first described by BEZUIDENHOUT *et al.* (1), provided a convenient source of antigen for use in serology. Since that time, several laboratories have developed IFAT on endothelial cells infected by *Cowdria* (34, 15, 23). Recently, partly because of high backgrounds obtained in an indirect ELISA, a competitive ELISA (CELISA) using a monoclonal antibody to the immunodominant antigenically conserved 32 kilodalton protein of *C. ruminantium* was described (16).

In this study, the development and the evaluation of an indirect ELISA aimed at being used as a single dilution test for the detection of antibodies to *C. ruminantium* in the serum of goats, sheep and cattle, is described.

MATERIALS AND METHODS

Sera

In Guadeloupe, 30 goats and 1 sheep were inoculated intravenously with 5 ml of supernatant of a bovine umbilical endothelial cell culture (BUE) infected with either the virulent Gardel stock (31), or an attenuated Senegal stock (MARTINEZ, unpublished) of *Cowdria*. Animals infected with the attenuated rickettsiae were not treated whereas those inoculated with a virulent stock were treated with oxytetracyclin (15 mg/kg/day IV for 2 or 3 days) as soon as their rectal temperature rose above 40 °C. Sera were collected at regular interval and preserved at - 20 °C until use. Eight goat sera, 2 cattle sera and 2 sheep sera were raised at Utrecht (The Netherlands) against the Senegal (14), the Welgevonden (7), the Kwanyanga (22) and the Lutale (14) stocks of *Cowdria* by the same technique. In addition, 10 sera from cattle, 10 sera from sheep and 2 sera from goats experimentally infected with either the Ball 3 (12), the Breed (8), the Kümm (6), the Kwanyanga, the Mali (19), the Mara (9) and the Welgevonden stocks of *C. ruminantium* were provided by the Onderstepoort laboratory, Republic of South Africa (10).

Negative sheep, goat and cattle sera were collected in regions without *Amblyomma* ticks and therefore free of

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cowdriosis (France, The Netherlands, Islands of Les Saintes in the West Indies), or in areas infected with *A. variegatum* but where heartwater has never been identified (Mayotte, 11 islands of the Lesser Antilles). These populations of negative sera were used for the calculation of the specificity of the test.

Experimental *Ehrlichia* infections

Infections with *Ehrlichia bovis* were monitored in 2 calves, *E. ovina* in 2 sheep and *E. phagocytophila* in one goat. Clinical manifestation of these experimental infections have been described in more detail elsewhere (17). The isolate of *E. bovis*, which caused moderate but prolonged fever in Friesian calves, originated from Kenya (26). The isolate of *E. ovina* from Turkey caused prolonged fever periods in Dutch sheep, which eventually recovered spontaneously. Finally, *E. phagocytophila*, causal agent of tick-borne fever of ruminants, was isolated from cattle on the North Sea Island of Ameland, The Netherlands (32). All isolates were stored in liquid nitrogen as infected stabilates, cryopreserved with 10 % DMSO before being used in this study. Aliquots of 2 ml deep-frozen blood were rapidly thawed and inoculated intravenously. The animals were monitored by taking their daily rectal temperature and clinical inspection. Sera were collected from all experimental animals once a week.

Antigen preparation

C. ruminantium (Gardel stock) was cultivated in bovine umbilical endothelial cell (BUE) cultures as previously described (23). When approximately 80 % of the cell monolayer was lysed, the remaining adherent cells were scraped, mixed with the supernatant and centrifuged at 2500 g for 15 min. The antigen was constituted by the pellet resuspended in sterile PBS and disrupted by 5 cycles of sonication (30 s each) interspersed by 1 min interval in an ethanol-dry ice bath. The antigen concentration was expressed in protein content which was determined by the method of Bradford.

ELISA procedure

An indirect ELISA method was used (11). Microplates (Nunc immuno-modules) were coated overnight at 37 °C with 5 µg/ml of antigen in carbonate-bicarbonate buffer 0.1 M, pH 9.5 (100 µl per well). For the assay, the plates were washed 3 times with phosphate buffer saline 0.1 M, pH 7.2, supplemented with 0.1 % tween 20 (PBS-tween, solution used for all washings). To each well was added 100 µl of test serum diluted in PBS-tween added with 3 % skimmed cow milk (Régilait). The optimal working dilutions of sera determined by checkerboard titration were found to be 1/800 for cattle, 1/400 for sheep and

1/400 for goats. The plates were incubated at 37 °C for 1 hour and washed 5 times. Rabbit anti-goat, anti-cattle or anti-sheep IgG conjugated to horse radish peroxidase optimally diluted in the blocking buffer was then added (100 µl per well) and the plates incubated at 37 °C for 1 hour. After 5 washings, each well was filled with 100 µl of citrate buffer 0.1 M, pH 5.5, containing 0.5 mg/ml O-phenylene diamine and 3 µl/ml of 9 % H₂O₂. The enzymatic reaction was stopped after 30 min of incubation at room temperature by adding 50 µl of 2N H₂SO₄ per well and the absorbance was read at 495 nm.

Sensitivity and specificity of the ELISA

To use the ELISA as a single dilution test, the threshold between positive and negative sera was determined at optimum working dilution of serum and conjugate for cattle, sheep and goats. The frequency distribution of absorbances of negative populations of sera were determined. Although these distributions were slightly skewed to the right, they were approximated normal distributions and the cut-off point value was fixed at mean absorbance of negative sera + 2.58 SD ($t = 2.58$ for $P < 0.01$, 24). The sensitivity and specificity of the ELISA were then calculated using these threshold values.

Precision of the ELISA

Intra and inter-assay variability were assessed using goat sera. Twelve replicates of 8 sera, the mean absorbance of which ranged between 0.28 and 1.1, were tested in each plate. One set of 3 identical plates was used each day for 3 days. The precision of the test was expressed in residual coefficient of variation (CV %) after computation of data using general linear procedures (29).

RESULTS

In the indirect ELISA described in this study, non specific binding of undesirable proteins was low (absorbance around 0.06) and the ratio between positive and negative sera was high, ranging between 3 and 20. Specific antibodies were detected between 9 and 19 days post-inoculation in all sera ($n = 31$) of animals experimentally infected with the Gardel and the Senegal stocks of *Cowdria* in Guadeloupe. The overall sensitivity of the ELISA calculated with the sera from Guadeloupe in addition to experimental sera from The Netherlands and the Republic of South Africa was 100 %. However, when field sera from RSA which were considered positive on the basis of the resistance of the animals to challenge were taken into account, the sensitivity of the ELISA ranged between 97.3 % and 98.6 % (table I).

TABLE I Sensitivity of the ELISA.

Animal specie	Origin of sera	No. of sera	No. positive sera	Sensitivity
Cattle	Rep. South Afr.	10	10	100
	Netherlands	2	2	
Goats	Rep. South Afr.	10 ¹	8/9	95.8/97.9
	Netherlands	8	8	
	Guadeloupe	30	30	
Sheep	Rep. South Afr.	10	10	100
	Netherlands	2	2	
	Guadeloupe	1	1	
Total general		73	71/72	97.3/98.6

¹ Eight sera were collected in the field and considered positive on the basis of the survival of the animal to challenge, the other 2 sera were from experimental infection.

The results on the specificity of the test are summarized in table II. The overall specificity calculated with 2,925 sera was 97 % indicating an overall percentage of false positive sera of 3 %. However, if we consider the 3 species separately, the specificity was far lower for sheep (5.6 % of false positive sera) than for goats and cattle (1.4 % and 2.2 % of false positive sera respectively). Because of the existence of positive sera in areas infec-

TABLE II Specificity of the ELISA.

Animal specie	Origin of sera	No. of sera	No. positive sera	Specificity
Cattle	Rep. South Afr.	5	1 (20)	80
	France	30	0 (0)	100
	Netherlands	10	0 (0)	100
	Mayotte	320	5 (1.6)	98.4
	West Indies	1028	25 (2.4)	97.6
	Total for cattle	1393	31 (2.2)	97.8
Goats	Rep. South Afr.	5	0 (0)	100
	Les Saintes (FWI)	67	1 (1.5)	98.5
	West Indies	579	8 (1.4)	98.6
	Total for goats	651	9 (1.4)	98.6
Sheep	Rep. South Afr.	5	0 (0)	100
	Netherlands	10	0 (0)	100
	Les Saintes	35	1 (2.9)	97.1
	West Indies	831	48 (5.9)	94.2
	Total for sheep	881	49 (5.6)	94.4
Total general		2 925	89 (3)	97.0

ted with *A. variegatum* but free of cowdriosis, the cross-reactivity with *Ehrlichia* species some of which are known to be transmitted by this tick (*E. bovis*), was investigated. A seroconversion was revealed by the ELISA on *Cowdria* antigen in 1 out of 2 cattle inoculated with *E. bovis* (figure 1) and in 2 sheep inoculated with *E. ovina* (figure 2). No crossreaction was observed in 1 sheep infected with *E. phagocytophila* (figure 2).

As shown in figure 3, the intra-plate variability expressed in CV % decreased when the absorbance of the serum increased. The values of intra and inter-assays variability were 7.5 % and 7.8 % respectively, indicating that within plate variations represented the main part of the variability.

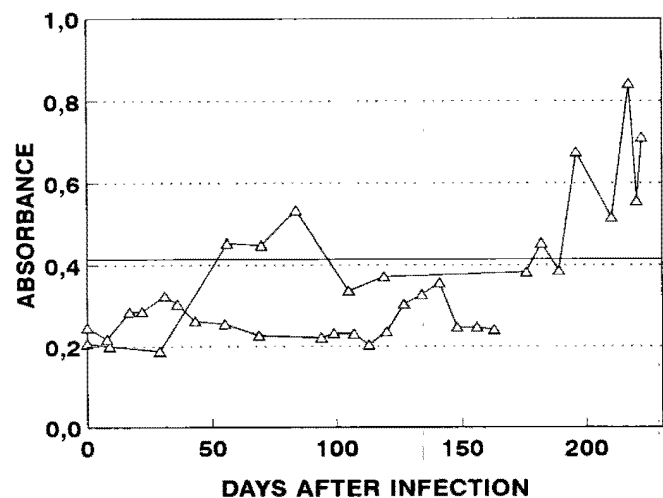


Figure 1. Kinetic of antibody levels of 2 cattle inoculated with *Ehrlichia bovis* as determined by ELISA on *Cowdria* antigen.

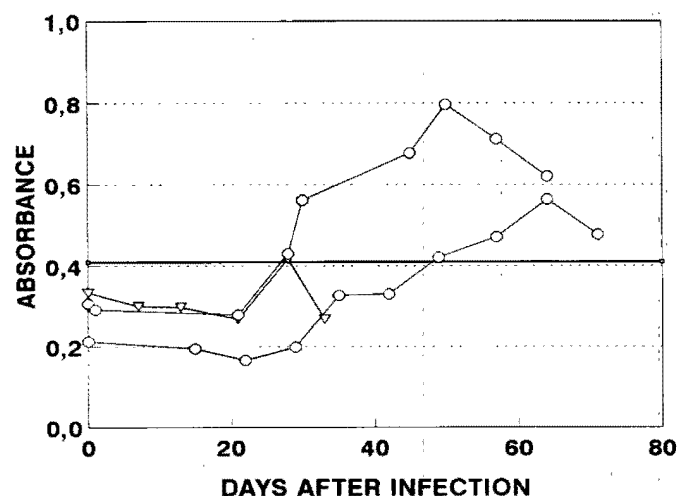


Figure 2 : Kinetic of antibody levels of 2 sheep inoculated with *Ehrlichia ovina* (O) and 1 sheep inoculated with *E. phagocytophila* (Δ) as determined by ELISA on *Cowdria* antigen.

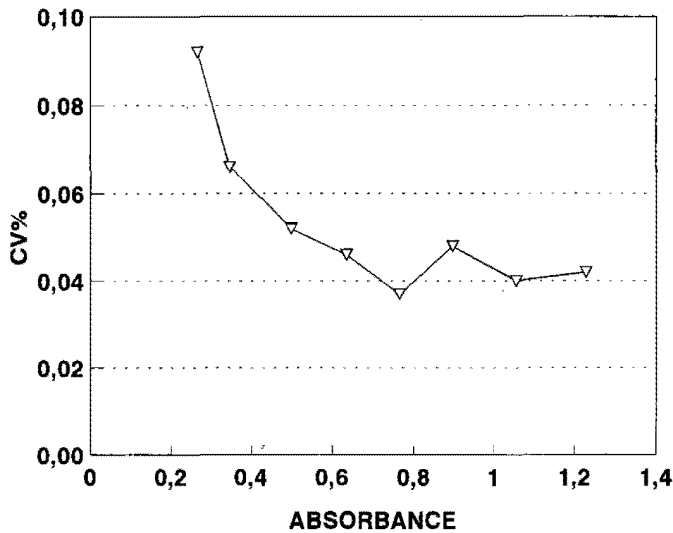


Figure 3 : Evolution of intra-plate variability of the ELISA expressed in residual coefficient of variation, as a function of the absorbance of the serum. Each point represents the mean of 72 measures.

DISCUSSION

In this study a single dilution indirect ELISA was described. Its reproducibility was good compared to other enzyme immunoassays (18). The variability of the measures was lower when the absorbance of the serum increased (figure 3), justifying the utilization of 8 sera ranging from low to high absorbance to evaluate the precision of the method. In contrast to other attempts in developing a similar ELISA, the problems of high backgrounds were easily overcome by using a convenient blocking agent such as skimmed cow milk.

The sensitivity was good, the ratio between positive and negative sera ranging between 3 and 20. The overall sensitivity calculated with 73 sera from sheep, goats and cattle varied between 97.3 % and 98.6 % (table I). However, amongst these 73 positive sera, the sera which were found negative (1 or 2 depending on the assay) were collected in the field from goats which were considered immune on the basis of their resistance to challenge (10). However, resistance to a virulent infection does not constitute an absolute criterion of previous contact with the parasite since populations of goats within a breed can acquire a genetically determined resistance under selective pressure. Thus, populations of creole goats from Guadeloupe isolated from an endemic area of cowdriosis for more than 10 years and reared in tick free conditions expressed a degree of resistance of 50 % (25). If only experimental sera are taken into account, antibodies to *Cowdria* were detected in 100 % of the samples between 9 and 19 days after inoculation. The time necessary for

the first antibodies to be detectable is in agreement with SEMU *et al.* (30). The existence of serotypes (15, 23) had no significant influence on the sensitivity of the ELISA which was able to detect antibodies in sera raised against 11 different stocks of *Cowdria* from all parts of Africa and from the Caribbean.

The calculation of the specificity revealed an overall percentage of false positive sera of 3 %. Previous studies conducted in the Caribbean have shown the existence of positive sera in islands where cowdriosis has never been identified in spite of active research (3). These positive sera identified for the first time by CAMUS (2) using IFAT performed on Kümm antigen were confirmed by IFAT on BUE cells infected with *Cowdria* (23) and by CELISA (27). The presence of seropositive ruminants in several islands from the Caribbean where cowdriosis probably does not occur, was confirmed in this study using the indirect ELISA (table II). In contrast, no positive serum was recorded amongst 50 sera from Europe. The specificity was good when the test was used on cattle or goat sera (97.8 % and 98.6 % respectively), but was only 94.4 % on sheep. The false positive sera were not uniformly spread in the Caribbean, but located mainly in 2 islands out of 11 tested (4). Since it is likely that heartwater does not exist in these 2 islands, crossreactivity with related microorganisms possibly associated with ticks should be considered. In this respect, crossreactivity with *Ehrlichia equi* and to a lesser degree *E. canis* has previously been shown by LOGAN *et al.* (20). As a matter of fact, in our study, crossreactivity between antisera to *Ehrlichia bovis* or *E. ovina*, and *Cowdria* antigen was revealed by the indirect ELISA. In contrast, there was no crossreaction with *E. phagocytophila*. These crossreactions had little influence when using the test to screen large quantities of goat and cattle sera in epidemiological surveys. However, in certain situations, the percentage of false positive sheep sera was too high. Serological data from epidemiological studies should therefore be interpreted carefully in this species in which there is a particular need for a more specific antigen. Besides epidemiological studies, the test proved very simple and useful to follow the antibody response of domestic ruminants in controlled experiments of vaccination.

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A solid phase enzyme immunoassay for the detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants was developed by using microorganisms cultivated on bovine umbilical endothelial cells as antigen. When the culture showed 90 % lysis, the supernatant was centrifuged, sonicated and coated on polystyrene microtiter plates. Antibodies were detected as early as 9 days after experimental immunization of goats. The sensitivity of the ELISA calculated with 73 ruminant sera ranged between 97.3 % and 98.6 %. The overall specificity of the test was 97 % (N = 2925). However, the specificity was far lower for sheep (94.4 %, N = 881) than for goats (98.6 %, N = 651) and cattle (97.8 %, N = 1393). Crossreactivity which could explain some of the false positive reactions, was found between *Cowdria* antigen and sera raised against *Ehrlichia bovis* (1 bovine positive out of 2 inoculated) or *E. ovina* (2 sheep out of 2 inoculated became positive) but not with *E. phagocytophila*. The intra-assay and inter-assay variability were 7.5 % and 7.8 % respectively, indicating a good reproducibility of the ELISA.

Key words : Ruminant - Cattle - Goat - Sheep - *Cowdria ruminantium* - Antibody - ELISA test - Antigen - Sera - *Ehrlichia bovis* - *Ehrlichia ovina* - *Ehrlichia phagocytophila*.

MARTINEZ (D.), COISNE (S.), SHEIKBOUDOU (C.), JONGEJAN (F.). Detección de anticuerpos de *Cowdria ruminantium* en el suero de rumiantes domésticos mediante el ELISA indirecto. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 115-120

Se desarrolló una fase sólida de inmunoensayo enzimático para la detección de anticuerpos de *Cowdria ruminantium*, en el suero de rumiantes domésticos. Como antígeno se usaron cultivos de microorganismos en células de endotelio umbilical bovino. Cuando se alcanzó una lisis de 90 % en el cultivo, se centrifugó el sobrenadante, se trató por ultrasonido y se cubrió con poliestireno en placas de microtitulación. Los anticuerpos se detectaron 9 días después de la inmunización experimental de las cabras. La sensibilidad del ELISA, calculada con 73 sueros de rumiantes, se localizó en un intervalo de 97,3 a 98,6 %. La especificidad general del test fue de 97 % (N = 2925). Sin embargo la especificidad fue inferior para las ovejas (94,4 %, N = 881) que para las cabras (98,6 %, N = 651) y los bovinos (97,8 %, N = 393). Las reacciones cruzadas, que podrían explicar algunos de los falsos positivos, se dieron entre el antígeno de *Cowdria* y los sueros preparados contra *Ehrlichia bovis* (1 bovino positivo de cada 2 inoculados) o *E. ovina* (2 ovejas de cada 2 inoculadas fueron positivas), pero no contra *E. phagocytophila*. Los ensayos de variabilidad intra-test y inter-test fueron de 7,5 % y 7,8 % respectivamente, indicando una buena reproductibilidad del ELISA.

Palabras claves : Rumiante - Bovino - Caprino - Ovino - *Cowdria ruminantium* - Anticuerpo - Test ELISA - Antígeno - Suero - *Ehrlichia bovis* - *Ehrlichia ovina* - *Ehrlichia phagocytophila*.